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Purpose. To examine the role of complement factor D (CFD) in age-related macular degeneration (AMD) by analysis of genetic association, copy number variation, and plasma CFD concentrations.

Methods. Single nucleotide polymorphisms (SNPs) in the *CFD* gene were genotyped and the results analyzed by binary logistic regression. *CFD* gene copy number was analyzed by gene copy number assay. Plasma CFD was measured by an enzyme-linked immunosorbent assay.

Results. Genetic association was found between *CFD* gene SNP rs3826945 and AMD (odds ratio 1.44; $P = 0.028$) in a small discovery case-control series (462 cases and 325 controls) and replicated in a combined cohorts meta-analysis of 4765 cases and 2693 controls, with an odds ratio of 1.11 ($P = 0.032$), with the association almost confined to females. Copy number variation in the *CFD* gene was identified in 13 out of 640 samples examined but there was no difference in frequency between AMD cases (1.3%) and controls (2.7%). Plasma CFD concentration was measured in 751 AMD cases and 474 controls and found to be elevated in AMD cases ($P = 0.00025$). The odds ratio for those in the highest versus lowest quartile for plasma CFD was 1.81. The difference in plasma CFD was again almost confined to females.

Conclusions. CFD regulates activation of the alternative complement pathway, which is implicated in AMD pathogenesis. The authors found evidence for genetic association between a *CFD* gene SNP and AMD and a significant increase in plasma CFD concentration in AMD cases compared with controls, consistent with a role for CFD in AMD pathogenesis. (*Invest Ophthalmol Vis Sci.* 2011;52:8828–8834) DOI:10.1167/iovs.11-7933

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Complement factor D (CFD; also known as adipsin) is a member of the chymotrypsin family of serine proteases and regulates a key step in the activation of the alternative complement pathway.[1,2] It is expressed in several tissues and cell types, but the major source of plasma CFD in humans is adipose tissue[3] (http://www.cgl.ucsf.edu/cgi-bin/genentech/genehub-gepis/), in which it is secreted by both mature adipocytes and macrophages.[4–7] Like other secreted serine proteases, the N-terminal signal peptide is first cleaved in the secretory pathway, leaving an inactive zymogen, proCFD.[1,8] However, its subsequent maturation differs from other serine proteases in two main ways. First, the five to six amino acid N-terminal activation domain in humans is rapidly cleaved, either within the secretary pathway or soon after its release into plasma, leaving >99% of plasma CFD in the CFD rather than proCFD form.[9–11] Second, activation is thought to occur subsequent to CFD formation as a result of a conformational change which occurs when it binds C3bB, a complex of factor B, magnesium ions, and complement component 3b (C3b).[1,2,8] CFD becomes transiently active and is able to cleave factor B within this complex into Ba and Bb fragments. This is widely regarded as a rate limiting step in formation of the C3bBb complex or C3 convertase, which amplifies the initial signal and is crucial to activation of the alternative complement pathway.[1,4] CFD has a uniquely narrow substrate specificity, only cleaving factor B. Plasma CFD concentrations are very low (1–2 μg/mL),[12] the lowest of any complement protein, strikingly lower than plasma C3 (1–2 mg/mL) or factor B (200μg/mL),[13] so that it only ceases to be limiting at 9 to 10 times higher concentrations.[11]

Age-related macular degeneration (AMD) is the most common cause of severe visual handicap in industrialized coun-

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tries.[14] It is associated with the progressive deposition of extracellular material (drusen or basal deposits) between the basal surface of the macular RPE and Bruch's membrane.[15] This is thought to be associated with immune attack, leading to dysfunction and eventual death of macular RPE cells (geographic atrophy; GA).[16] In 5%–10% of affected subjects, choroidal neovascularization (CNV) can lead to hemorrhage and exudation within the macula, causing catastrophic loss of vision. The major risk factors in AMD include age, smoking, and genetic influences.[17] The latter include genetic variation in genes influencing the alternative complement pathway such as CFH, C2/BF, C3, and FI (reviewed in Refs. 16, 18). Other genetic risk factors include variants influencing extracellular matrix function, such as *TIMP3*, *FBL6*, and *ARMS2* and genes involved in lipid metabolism, such as *APOE*, *LIPC*, and *CETP*.[16,18–20]

The strong association of AMD with genes encoding components of the alternative complement pathway prompted us to examine the role of genetic variation influencing CFD, including copy number variation and plasma concentrations, particularly because CFD has an important role in activation of this pathway.

Materials and Methods

Study Cohorts

The clinical and demographic features of the six case-control series are summarized in Table 1.

Genotyping

Initially, only three SNPs were identified within the *CFD* gene (rs1683564, rs3826945, rs1683563) one of which (rs16853564) was not successfully genotyped. DNA was genotyped in two UK case-control series (UK1, UK2) for two intronic *CFD* SNPs, rs3826945 and rs1683563, using genotyping technology (TaqMan; Applied Biosystems, Foster City, CA). All genotyping assays were pre-validated by the supplier. Five-microliter reactions were set up in 384-well plates (TaqMan Universal PCR Master Mix, No AmpEraseUNG; Applied Biosystems) with 7.5 ng DNA, 1 μM of each primer, and 0.2 μM of probe. The thermal cycling reactions (95°C for 10 minutes, followed by 40 cycles at 92°C for 15 seconds and 60°C for 1 minute) were run and analyzed (7900HT Sequence Detection System; Applied Biosystems) with genetic analysis software (Genotyper SDS system, version 2.2; Applied Biosystems). As controls, each plate contained multiple blank wells without DNA. Similarly, for the USA 2 (University of Pennsylvania) and Dutch-German (Nijmegen) case-control series, assays (TaqMan; Applied Biosystems) were also used to genotype rs3826945 and rs1683563. In the USA 3 series, SNPs rs3826945 and rs1683563 were genotyped using a different assay (Sequenom iPLEX; Sequenom, San Diego, CA), as described.[21]

In the USA 1 case-control series (University of Michigan), *CFD* SNPs rs1683563 and rs3826945 were genotyped as follows. Primers were designed and used to polymerase chain reaction (PCR) amplify the amplicons for dye-termination Sanger sequencing. The primers for the rs1683563 were: forward primer 5′AGTGTGGCCTTCTCCGACAG and reverse primer 5′AAATCTCTCCTGCTGCACTGA. Primers for rs3826945 were: forward primer 5′CACGTGTTAGACCCCCTCAC and reverse primer 5′TGGAAGAGCAGGAATGAGGT. Standard PCR conditions were followed with the use of polymerase (TaKaRa Ex Taq; Takara Bio Inc., Shiga, Japan) with 1 μL of DNA (approximately 100 ng/μL), and PCR conditions: melting temperature 94°C for 2 minutes; 35 cycles at 94°C for 30 seconds; annealing at 60°C for 30 seconds; extension at 72°C for 30 seconds; followed by extension at 72°C for 7 minutes. PCR products were run on 1.5% ethidium bromide agarose gels and viewed on a gel documentation system (Kodak 440; Kodak, Rochester, NY). Aliquots of the PCR products were submitted for sequencing at the University of Michigan core facility.

Copy Number Variation Analysis

Copy number variation at the CFD locus was assessed using a copy number assay (Hs01536182_cn; TaqMan; Applied Biosystems), run simultaneously with a copy number reference assay (TaqMan; Applied Biosystems) for RNAseP in a duplex real-time polymerase chain reaction. The CFD probe was FAM-labeled and the endogenous control was VIC-labeled. Ten-microliter reactions were set up in 384-well plates (TaqMan Universal PCR Master Mix, No AmpEraseUNG; Applied Biosystems) with 10 ng genomic DNA, 1 μM of each primer, and 0.2 μM of probe. The thermal cycling reactions (95°C for 10 minutes, followed by 40 cycles at 92°C for 15 seconds and 60°C for 1 minute) were run on a sequence detection system (7900HT Sequence Detection System;

Table 1. Cohort Characteristics

Cohort	Status	*n*	%	Grading	%	Mean Age (y)	SD	Sex			
								Male	%	Female	%
Scottish	Control	347	41.0			78.0	8.5	152	43.8	199	57.3
	Case	499	59.0	Late AMD	48.3	77.9	9.2	190	38.1	315	63.1
	Total	846									
English	Control	421	32.1			75.6	7.7	171	40.6	232	55.1
	Case	891	67.9	Late AMD	100.0	78.7	7.2	377	42.3	472	53.0
	Total	1312									
USA 1	Control	311	33.8			76.6	5.1	143	46.0	168	54.0
	Case	608	66.2	Late AMD	85.9	79.3	7.3	222	36.5	386	63.5
	Total	919									
USA 2	Control	378	42.1			75.6	7.9	177	46.8	203	53.7
	Case	520	57.9	Late AMD	100.0	80.0	8.5	206	39.6	314	60.4
	Total	898									
USA 3	Control	887	40.1			75.4	6.1	394	44.4	501	56.5
	Case	1326	59.9	Late AMD	100.0	80.7	6.3	583	44.0	758	57.2
	Total	2213									
Dutch-German	Control	562	31.9			72.7	6.6	245	43.6	314	55.9
	Case	1201	68.1	Late AMD	91.3	75.9	8.2	456	38.0	744	61.9
	Total	1763									

Grading was performed by an ophthalmologist and refers to the worst eye. Controls were also examined. Late AMD includes cases of GA and neovascular AMD (CNV). Grading was based on the Clinical Age-Related Maculopathy Grading System (CARMS).[33] All cohorts were made up of Caucasian individuals. The numbers shown in the main text refer to the number of individuals that were successfully genotyped for *CFD* SNPs.

Applied Biosystems). Samples were analyzed in triplicate, and each plate contained multiple "no template" control wells without DNA. The relative copy number of CFD, normalized to the known copy number of the RNAseP reference sequence, was calculated using commercially available software (Copycaller v1.0; Applied Biosystems).

Plasma Samples

Blood samples were collected in dipotassium EDTA-coated tubes. Plasma was separated from blood cells by centrifugation within 3 hours of collection and was frozen in aliquots at −80°C until use.

Measurement of CFD in Plasma by ELISA

Plasma CFD was measured using an ELISA development kit (DuoSet) for human complement factor D (R&D Systems, Minneapolis, MN), optimized for use in plasma. The kit was used as described by the manufacturer; plasma samples were diluted 1 in 4000 for analysis. A standard curve ranging from 0 to 2.5 ng/mL was included on each plate, alongside three internal control plasma samples used to assess interassay variability. The interassay coefficient of variation was below 10%. CFD was measured without prior knowledge of disease status for each sample. AMD cases and control samples were included on each plate assayed and all samples were measured in duplicate.

Statistical Analysis

Genotyping, copy number variation, and CFD ELISA data were maintained using statistical analysis and data management software (SPSS version 17; SPSS Inc., Chicago, IL).

SNP genotyping was assessed for deviation from Hardy-Weinberg equilibrium in AMD cases and controls using a χ^2 test. The association of SNPs with AMD was tested using logistic regression analysis to model the probability of disease occurrence, including known risk factors—age, sex, and smoking history—as variables along with SNP genotype in the analyses. Age was included as a continuous variable. Sex and smoking history (ever/never) were considered as categorical values. A P value of < 0.05 was considered a significant association with AMD, as correction for multiple testing was not necessary. Logistic regression was also performed in individual and combined cohorts stratified by sex. In these analyses, SNP genotype, age, and smoking status were included as variables. Odds ratios (ORs) and 95% confidence intervals (CIs) for association were also calculated. A Forest plot for ORs and 95% CIs for each cohort was constructed with software (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA).

Association of AMD with copy number variation (defined as either deletion or duplication) at the *CFD* locus was evaluated using Fisher's exact test.

The Mann-Whitney U test was used to assess differences between median values of CFD measured in plasma for AMD cases (GA, CNV, and total AMD) and controls. Logistic regression, controlling for age (< 65 years of age/65 to 80 years of age, and aged over 80 years), sex, and smoking (ever/never), was performed to compare the first and fourth quartiles of plasma CFD values in AMD cases and controls, and to calculate ORs and 95% CIs. Body mass index (BMI; <25, 25–29.9, >30) was included as a covariate in analysis of the UK 2 cohort.

Results

Genetic Association between CFD Variants and AMD

Initially we examined the association of AMD with SNPs within the *CFD* gene. Only three common *CFD* SNPs (rs 3826945, rs1683564, and rs1683563) with a minor allele frequency >10% in the CEPH Caucasian reference population were identified in HapMap. One of these, rs16853564, was not successfully genotyped. Other SNPs had lower minor allele frequencies and so were unlikely to provide adequate power. The Tagger-pairwise Tagging algorithm (http://hapmap.ncbi.nlm.nih.gov/) identified rs3826945 and rs1683564 as tagging SNPs. Rs3826945 and rs1683563 are in high linkage disequilibrium (LD) (D' = 1), but not complete LD ($r^2 = 0.314$), so both SNPs remain informative. A dominant genotypic model and binary logistic regression analysis was used, including sex, smoking, and SNP as categorical variables and exact age as a continuous covariate.

A Scottish case-control series (UK 1) was examined initially, consisting of 462 genotyped AMD cases, 52% of whom had early AMD, and 48% had late AMD (GA or CNV) and 325 examined controls. The rs1683563 SNP was not significantly associated with AMD ($P = 0.07$). The rs3826945 SNP however showed evidence of association with AMD, particularly in females, who showed an odds ratio of 1.70 (95% CI, 1.11–2.63; $P = 0.016$), although the combined sexes also showed a significant odds ratio of 1.44 (95% CI, 1.04–2.00; $P = 0.028$) (Table 2).

Table 2. SNP Association Results for *CFD* SNP rs3826945

Cohort	Cases (*n*)	Controls (*n*)	OR	95% CI	*P* Value
UK1					
Females	287	182	1.7	1.11–2.63	0.016
Males	175	143	1.22	0.74–2.03	0.432
All	462	325	1.44	1.04–2.00	0.028
UK 2, USA 1, USA 2					
Females	1133	576	1.36	1.11–1.68	0.004
Males	779	469	1.07	0.85–1.36	0.571
All	1865	1029	1.23	1.05–1.43	0.01
USA 3, Dutch-German					
Females	1409	736	1.03	0.85–1.24	0.801
Males	976	580	1.01	0.82–1.24	0.92
All	2385	1316	1.01	0.88–1.16	0.922
Combined cohorts					
Females	2833	1497	1.18	1.04–1.34	0.012
Males	1932	1196	1.04	0.90–1.21	0.57
All	4765	2693	1.11	1.01–1.23	0.032

A dominant model was used to assess the association of the minor allele of rs3826945 to AMD, controlling for age, sex, and smoking in a total of six cohorts, comprising the discovery cohort, and two further replication groups. UK 1, UK 2, USA 1, and USA 2 cohorts were also genotyped for rs1683563, which was not significantly associated with AMD when included as a covariate in the analysis of these cohorts.

In view of the low power to reliably detect such a small effect, we sought to replicate this result by genotyping both SNPs in a larger case-control set, consisting of an additional UK series (UK 2) (773 genotyped cases—all with severe AMD—and 362 examined controls) and two north American series (USA 1, USA 2). USA 1 (University of Michigan) included 580 genotyped cases (86% severe AMD) and 296 examined controls while the USA 2 (University of Pennsylvania) series included 512 genotyped cases (100% severe AMD) and 371 examined controls. The rs1683563 SNP was not significantly associated with AMD ($P = 0.89$). The results of binary logistic regression using age, sex, smoking, and the *CFD* rs3826945 SNP as variables in 1865 cases and 1029 controls showed a significant association between AMD and rs3826945, with an odds ratio of 1.23 (95% CI, 1.05–1.43; $P = 0.01$).

A second replication group was then tested, which included two further large case-control series, one from USA (USA 3, Tufts University) and one from Europe (Dutch-German series). The USA 3 series consisted of 1211 genotyped cases (all with severe AMD) and 790 examined controls, while the Dutch-German series consisted of 1174 genotyped cases (68% with severe AMD) and 526 examined controls. The results of this analysis of 2385 cases and 1316 controls showed no evidence of association with rs3826945, because the odds ratio was 1.01 (0.88–1.16) and *P* value 0.922. The minor or risk allele frequency (C allele) for rs3826945 varied from 0.31 to 0.35 in individual case-control series but was very similar in the UK 1 (0.34), first replication series (0.33) and second replication series (0.32), so is an unlikely explanation for the apparent differences between cohorts (Table 1).

We combined all the above results into a single meta-analysis (4765 cases and 2693 controls), which showed a significant association overall (OR, 1.11; CI, 1.01–1.23; $P = 0.032$) between AMD and rs3826945. The odds ratio was significant in females (OR, 1.18; 95% CI,1.04–1.34 in females; $P = 0.012$) but not in males (OR, 1.04; 95% CI, 0.90–1.21; $P = 0.57$). Similar results were obtained using an allelic (additive) model (data not shown). The results of the meta-analysis are summarized as a Forest plot in Figure 1 and Table 2. Interactions between smoking and *CFD* SNP rs3826945, or between body mass index and rs3826945, did not influence the association with AMD in cohorts where these data were available.

The minor allele of rs3826945 was not significantly associated with either GA or CNV in any of the series analyzed independently. In the combined series, there was a borderline significant association between the minor allele and CNV ($P = 0.043$; OR, 1.154; 95% CI, 1.004–1.325), but not with GA ($P = 0.359$; OR, 1.089; 95% CI, 0.908–1.306). This analysis included 1681 controls, 660 GA cases, and 1687 CNV cases. Splitting the combined series by sex, there was no significant association observed in males (755 controls, 266 GA cases, and 659 CNV cases), and there was only suggestively significant association in females (926 controls, 394 GA cases, and 1028 CNV cases). For GA females, the minor allele *P* value was 0.098 (OR, 1.223; 95% CI, 0.963–1.552); for CNV females, the *P* value was 0.056 (OR, 1.196; 95% CI, 0.995–1.437). The GA group is however much smaller than the CNV group, reducing the power to detect effects of CFD on these clinical subgroups. Plasma CFD is therefore significantly elevated in both neovascular and atrophic AMD, although it is more significant in neovascular AMD (but again, there are more cases).

Copy Number Variation in the *CFD* Gene

Copy number variation in the *CFD* gene was measured using a copy number assay (TaqMan; Applied Biosystems), in a duplex real-time polymerase chain reaction, as described in Materials and Methods. The relative copy number of *CFD* was normalized to the known copy number of the RNAseP reference sequence and calculated using software (Copycaller; Applied Biosystems) using a conservative C_T threshold value of 32 across all plates. Copy number was measured in 311 AMD cases and 329 controls from the UK 1 (Scottish AMD) case-control series. Copy number variation in the *CFD* gene was identified in 4 out of 311 AMD cases (two with a single copy and two with three copies) and 9 out of 329 controls (one with zero copies, five with a single copy, and three with three copies). There was no significant difference in *CFD* duplication/deletion frequency between cases and controls (1.3% of AMD cases and 2.7% of controls; $P = 0.51$ using Fisher's exact test). The results are summarized in Figure 2.

Plasma CFD Concentration in AMD Cases and Controls

An enzyme-linked immunosorbent assay (ELISA) was developed to measure plasma CFD concentrations, as described in Materials and Methods. The plasma CFD concentration was measured in 751 AMD cases and 474 controls from the UK 1 and UK 2 case-control series and found to be elevated in AMD cases compared with controls; median plasma CFD concentration was 2.31 ± 0.043 (SEM) $\mu g\ mL^{-1}$ in cases and 2.08 ± 0.046 $\mu g\ mL^{-1}$ in controls ($P = 0.00025$; Fig. 3). When samples were broken down by sex, plasma CFD was significantly greater in AMD females than control females ($P = 0.0004$) but there was no significant difference between AMD males and control males ($P = 0.135$). The odds ratio for those of both sexes in the highest versus lowest quartile of plasma CFD was 1.81 (95% CI, 1.27–2.57; $P = 0.001$), while for the sexes

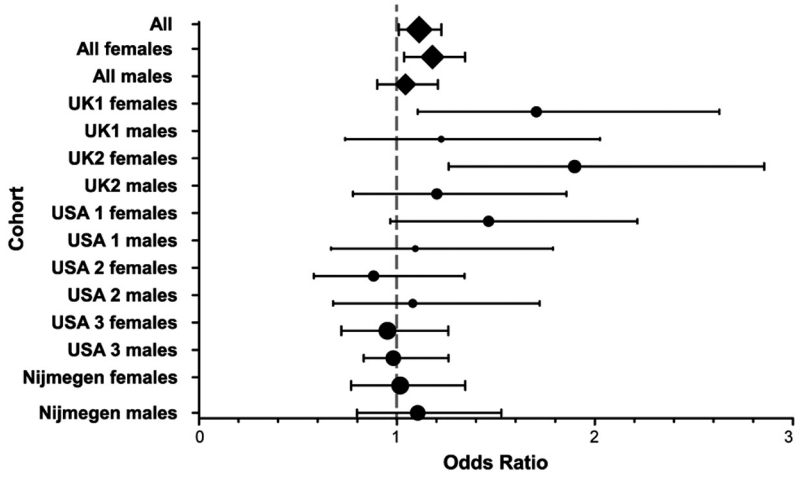

FIGURE 1. Forest plot summarizing the results of the meta-analysis of association studies between *CFD* SNP rs3826945 and AMD in three United States AMD case-control series (USA 1–3) and three European case-control series (UK 1–2, and Dutch-German [Nijmegen]). The result of meta-analysis showed a significant association in the combined sexes (OR, 1.11; $P = 0.032$) and in combined females (OR, 1.18; $P = 0.012$) but not in males.

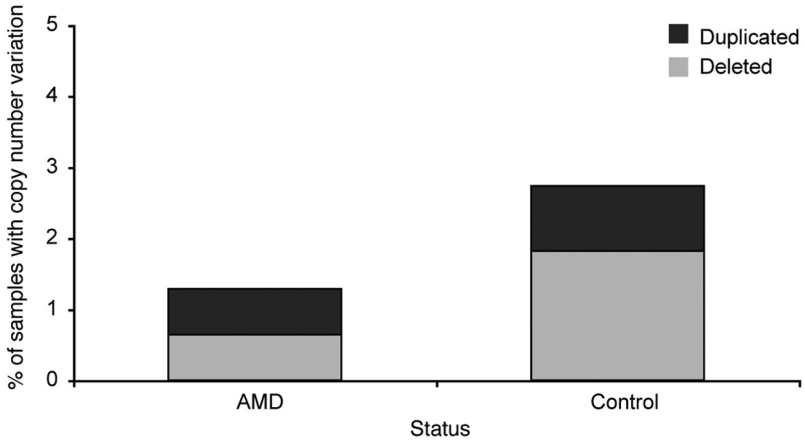

FIGURE 2. CNV in the *CFD* gene in AMD cases and controls showing the proportion of duplicated and deleted genes. The difference between AMD cases and controls was not statistically significant.

separately, the corresponding odds ratios were 2.15 (95% CI, 1.35–3.44; $P = 0.001$) in females and 1.42 (95% CI, 0.82–2.47; $P = 0.215$) in males.

There was no association between genotype at rs3826945 and plasma CFD concentration ($P = 0.309$). However, plasma CFD was found to be elevated in individuals with BMI >30 who had a median plasma concentration of 2.11 ± 0.076 (SEM) μg mL^{-1}, compared with BMI <25 (median value 1.77 ± 0.064 (SEM) μg mL^{-1}; $P = 3 \times 10^{-6}$). When BMI was included as a covariate in regression analysis for 272 controls and 402 AMD cases for whom BMI data were available, the odds ratio for the highest versus lowest quartile of plasma CFD was 2.03 (95% CI, 1.26–3.27; $P = 0.004$).

DISCUSSION

Complement factor D is a unique member of the alternative complement pathway both in terms of its unusual mode of activation and its tissue expression profile.[1,2] Adipose tissue is the major source of plasma CFD, which is thought to be constitutively secreted at a high rate but rapidly catabolized in tissues such as the kidney.[1,4] The fractional catabolic rate of plasma CFD has been estimated to be 60% per hour,[22] contributing to its low serum concentrations. White adipose tissue is increasingly regarded as an endocrine organ, secreting a variety of hormones, such as leptin, as well as immune and inflammatory mediators, such as CFD and tumor necrosis factor-α.[23,24] Adipose tissue contains several different cell types, the most abundant being adipocytes, but bone-marrow derived macrophages are also recruited in substantial numbers, possibly by chemokines such as monocyte chemoattractant protein-1 (MCP-1) that are expressed by adipocytes. The liver is the major source of most complement components found in plasma, with the notable exceptions of CFD and C1q.[25] However, adipocytes are also able to synthesize many complement components, particularly those involved in the initial steps of complement activation via both classical and alternative pathways (e.g., factor B, C2, C3, C4, C1Q, C1R, C1S) but they

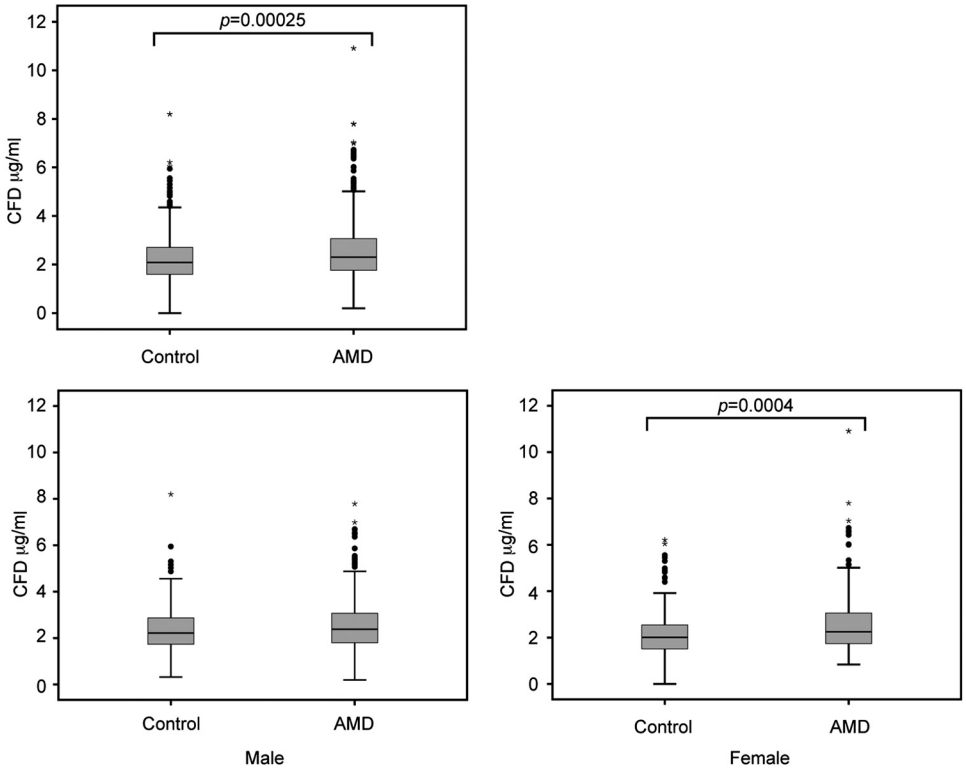

FIGURE 3. ELISA assay of plasma CFD concentration in AMD and control subjects in the combined UK 1 and UK 2 series. The difference was statistically significant both in the combined sexes ($P = 0.00025$; *upper panel*) and in females ($P = 0.0004$) but not in males.

express almost none of the terminal complex components (C5, C8A, C8B, C8G, C9), with the exception of C7.[5,26,27] Adipocytes therefore appear to be the major source of plasma CFD in normal individuals, which is supported by our finding that median plasma CFD was significantly higher in obese individuals compared with those with a BMI <25 ($P = 0.000003$), but cells in the monocyte/macrophage lineage also contribute.[4,25,26] The situation in individuals with diseases associated with alternative complement pathway activation, such as AMD, may not be identical.

The finding that plasma CFD is significantly raised (11% increase; $P = 0.00025$) in a large sample of AMD cases compared with disease-free controls is potentially important because it is widely thought to be rate-limiting in activation of the alternative complement pathway. Scholl et al.[28] reported a 33% increase in plasma CFD in a smaller sample of 112 AMD cases (92% with severe AMD) compared with 67 controls ($P < 0.001$). These authors also observed increases in plasma levels of complement components Ba and C3d, indicating chronic complement activation, perhaps spilling over into the systemic circulation from the eye. While Reynolds et al.[29] also found raised complement activation products in plasma, including Bb and C5a, they found no significant change in plasma CFD in a sample of 120 AMD cases (all with severe disease) compared with 60 controls, although odds ratios for plasma CFD were 1.4 and 3.1 respectively in models with and without genetic variants associated with AMD. The present study has substantially greater power to detect a small difference in CFD between cases and controls (751 cases and 474 controls) and therefore helps to resolve this issue. The majority (82%) of the combined UK 1 and UK 2 cases had severe AMD (GA or CNV), so disease severity is unlikely to influence the comparison between studies. Although BMI appears to be significantly associated with plasma CFD, BMI alone does not explain the significant association of plasma CFD level with AMD in our data, because including it as a covariate did not result in loss of the association.

The finding of raised plasma CFD in AMD versus controls begs the question as to the source of raised plasma CFD in AMD. Nonadipose sources of plasma CFD production reported to date include circulating monocytes/macrophages, lung, muscle, synovial tissue, and brain astrocytes.[4,25] In the eye, choroidal cells express CFD at over 10-fold higher levels than either neural retina or RPE, on the basis of quantitative real-time PCR (qPCR) analyses.[30] All initial components of both the alternative and classical complement pathways and most of the complement regulators (CFH, DAF, CFI) are mainly expressed in the choroid rather than in RPE or neural retina, although the precise cellular source is unclear. C3 levels in choroid-RPE are particularly high; approximately 5% of those in adult liver, and most C3 immunoreactivity is localized to the choriocapillaris.[30] In contrast, the terminal complement components are absent from choroid, RPE and neural retina, suggesting that these are provided by the blood.[30] Candidate choroidal sources of alternative complement pathway components such as CFD include macrophages and the choroidal capillaries adjacent to Bruch's membrane.

The possibility that AMD therapy could account for the observed changes in plasma CFD was considered but we do not have information on who was currently undergoing anti-VEGF therapy at the time of sampling. Changes in VEGF can result from activation of the alternative complement pathway but we are not aware that the reverse can occur, so there is no theoretical reason why such treatment would influence our results.

The colocalization of CFD and its substrates C3 and factor B in choroidal tissue need not imply local complement activation, which requires their deposition on a surface in which they can evade control by CFH, CFI, DAF, MCP, and complement receptors.[31] In AMD, most components of both the classical and alternative complement pathways and their regulators are deposited between RPE and Bruch's membrane in drusen, the hallmark deposits of early-stage AMD.[30] CFD deposition is not a feature of drusen, perhaps because of its very low plasma concentration but raised plasma CFD concentration in AMD compared with control subjects could reflect a high level of complement activation occurring in the choriocapillaris-Bruch's membrane-RPE region, rather than a systemic disorder of complement activation.

This is the first report of genetic association between AMD and variation within the *CFD* gene. The association is confined to a single noncoding SNP, rs3826945, within intron 4 of the *CFD* gene (chr19:813,912), which spans 3.9 kb on 19p13.3 and contains five exons. This variant is unlikely to directly influence CFD activity but is most likely to be in LD with a nearby functional variant(s). The region is one of high gene density, high recombination, and relatively low LD (http://hapmap.ncbi.nlm.nih.gov) perhaps explaining why the association does not appear to extend far outside the rs3826945 region.

The association was confirmed in a large meta-analysis, including 4765 predominantly severe AMD cases and 2693 examined controls. The effect size was very small (odds ratio 1.11) and almost confined to females (Table 1, Fig. 1). The sex difference is unlikely to result from the smaller male sample size—if this were true, the overall *P* value for the SNP in combined males and females ($P = 0.032$) should be more significant than in females only ($P = 0.012$). The same is true of the plasma CFD measurements. Multinomial logistic regression was performed using a custom/stepwise approach to investigate interaction between rs3826945 and sex. In agreement with the results obtained by regression analysis performed in females only, females appear to be more susceptible to AMD when they carry the risk allele of rs3826945 with an odds ratio (OR) for the interaction of 1.304 (95% CI, 1.142–1.489; $P = 8.73 \times 10^{-5}$) which was increased from OR 1.236 (95% CI, 1.117–1.367) for females without accounting for genotype, but not significantly so. Sex differences in the heritability of complex traits are common[32] so the sex-specific nature of the *CFD* association is both biologically plausible and interesting in light of the sex-specific changes in plasma CFD, both involving an effect more or less confined to females. This could suggest that the *CFD* SNP association serves to indicate an as yet unidentified variant regulating plasma CFD levels in females that is in linkage disequilibrium with rs3826945. No significant association was found between the *CFD* SNP (res3826945) and plasma CFD concentration but our study almost certainly lacked power to detect such an effect, given that almost 5000 cases were required to show the genetic association with AMD. If one or more *CFD* variant does influence plasma CFD directly (in females), then a causal effect on AMD risk, rather than raised plasma CFD being a consequence of AMD, has to be considered.

BMI does not explain the raised plasma CFD in AMD cases but if the proposed effect of *CFD* genotype on AMD is mediated by differences in plasma CFD (both of which are largely confined to females), it is possible that the observed variability in replication of the genetic association across case-control series may have been due to differences in BMI between populations because BMI data were only available in some series (UK 2, USA 1, Dutch-German). The lack of a statistically significant interaction between SNP and BMI argues against this, although the analysis may again have been underpowered.

Manipulation of plasma CFD concentration or inhibition of its activation in individuals at high genetic risk of AMD progression might be therapeutically useful regardless of whether the plasma CFD association is a cause or a consequence of the

disease. Further work is therefore required not only to confirm the genetic association but also to identify candidate regulatory variants by re-sequencing and functional studies of SNP effects on CFD expression in adipocytes or macrophages.

Follow-up of our findings, particularly investigating the relationship between rs3826945 and putative regulatory variants in its vicinity, may have therapeutic implications because of the important role of CFD in regulation of the alternative complement pathway. The proposed genetic association between *CFD* gene variants and AMD requires further investigation in large meta-analyses and, if confirmed, adds to the evidence implicating this pathway in AMD pathogenesis.

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